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Title: Compounds useful for the treatment of diseases responsive to antiangiogenetic therapy.

IPC: -

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09 September 2003

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PATENT- OG VAREMÆRKESTYRELSEN

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PVS

**COMPOUNDS USEFUL FOR THE TREATMENT OF  
DISEASES RESPONSIVE TO ANTIANGIOGENETIC THERAPY**

**TECHNICAL FIELD**

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This invention relates to the use of certain compounds for the treatment of diseases that are responsive to antiangiogenetic therapy, in particular for anti-metastatic treatment.

10

**BACKGROUND ART**

Angiogenesis (or neovascularisation) is the formation of new blood vessels by sprouting from preexisting vessels. Angiogenesis is generally absent in healthy adult or mature tissue. However, it occurs in the healthy body for healing wounds and for  
15 restoring blood flow to tissues after injury or insult. In females, angiogenesis also occurs during the monthly reproductive cycle and during pregnancy. Under these processes, the formation of new blood vessels is strictly regulated.

In many serious disease states, the body loses control over angiogenesis. Excessive angiogenesis occurs in diseases such as cancer, diabetic blindness, age-  
20 related macular degeneration, rheumatoid arthritis, and psoriasis. In these conditions, new blood vessels feed diseased tissues, destroy normal tissues, and in the case of cancer, the new vessels allow tumor cells to escape into the circulation and lodge in other organs (tumor metastasis).

Experimental evidence has accumulated over the years to show that a variety of  
25 strategies that limit angiogenesis also slow or inhibit tumour growth, suggesting that blocking tumour-induced angiogenesis is a valid, novel approach to tumour therapy.

However, there is a continued need for new antiangiogenetic therapies aiming at halting new blood vessel growth.

WO 98/47879 and WO 00/24707 (NeuroSearch A/S) describe a number of  
30 substituted phenyl derivatives active as chloride channel blockers.

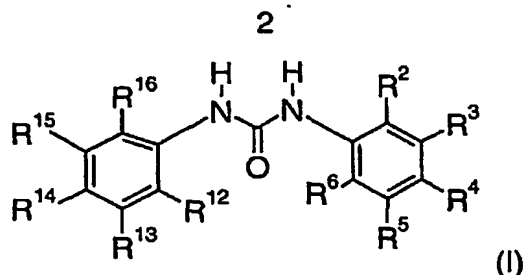
WO 00/76495 (Smithkline Beecham Corp.) describes a number of substituted phenyl derivatives active as IL-8 receptor antagonists.

**SUMMARY OF THE INVENTION**

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It is an object of the invention to provide new therapies for treating diseases that are responsive to antiangiogenetic therapy. In particular, it is an object to provide therapies for arresting tumor growth and preventing the formation of metastases.

In its first aspect, the invention provides the use of a compound of general  
40 formula I



or a pharmaceutically acceptable salt thereof

for the manufacture of a pharmaceutical composition for the treatment, prevention or alleviation of a disease or a disorder or a condition of a mammal, including a human,

5 which disease, disorder or condition is responsive to inhibition of angiogenesis.

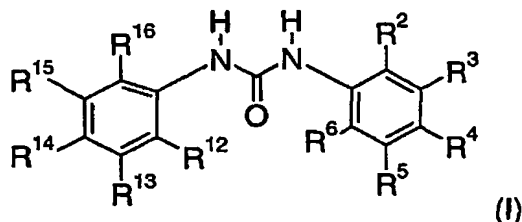
Other objects of the invention will be apparent to the person skilled in the art from the following detailed description and examples.

### DETAILED DISCLOSURE OF THE INVENTION

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According to the invention it has now been found that certain compounds can be used for the treatment of diseases that are responsive to antiangiogenetic therapy, in particular for anti-metastatic treatment.

Thus, in its first aspect, the invention relates to the use of a compound of general  
15 formula I



or a pharmaceutically acceptable salt thereof

wherein R<sup>2</sup> represents tetrazolyl;

R<sup>3</sup>, R<sup>4</sup>, R<sup>5</sup>, R<sup>6</sup>, R<sup>12</sup>, R<sup>13</sup>, R<sup>14</sup>, R<sup>15</sup>, and R<sup>16</sup> independently of each other represent  
20 hydrogen, halogen, trifluoromethyl, nitro, or phenyl;

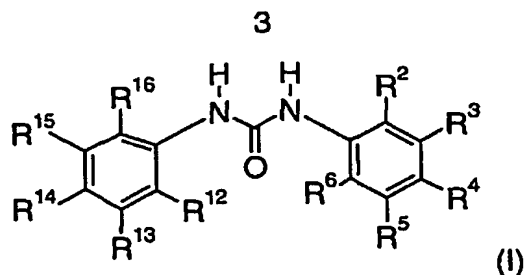
which phenyl is optionally substituted with halogen, trifluoromethyl, nitro, or -CO-NHR<sup>a</sup>;

wherein R<sup>a</sup> is hydrogen, alkyl, or phenyl;

for the manufacture of a pharmaceutical composition for the treatment, prevention or alleviation of a disease or a disorder or a condition of a mammal, including a human,

25 which disease, disorder or condition is responsive to inhibition of angiogenesis.

In another aspect the invention relates to a method of treatment, prevention or alleviation of a disease or a disorder or a condition of a living animal body, including a human, which disorder, disease or condition is responsive to inhibition of angiogenesis, comprising the step of administering to such a living animal body, including a human,  
30 in need thereof a therapeutically effective amount of a compound of general formula I



or a pharmaceutically acceptable salt thereof

wherein  $R^2$  represents tetrazolyl;

$R^3$ ,  $R^4$ ,  $R^5$ ,  $R^6$ ,  $R^{12}$ ,  $R^{13}$ ,  $R^{14}$ ,  $R^{15}$ , and  $R^{16}$  independently of each other represent  
 5 hydrogen, halogen, trifluoromethyl, nitro, or phenyl;  
 which phenyl is optionally substituted with halogen, trifluoromethyl, nitro, or  $-CO-NHR^a$ ;  
 wherein  $R^a$  is hydrogen, alkyl, or phenyl.

The living animal body to be treated according to this invention is preferably a mammal, most preferably a human, in need for such treatment.

10 In one embodiment of the compound of general formula I,  $R^2$  represents tetrazolyl;  $R^3$ ,  $R^4$ ,  $R^5$ ,  $R^6$ ,  $R^{12}$ ,  $R^{13}$ ,  $R^{14}$ ,  $R^{15}$ , and  $R^{16}$  independently of each other represent hydrogen, halogen, trifluoromethyl, or nitro.

In a second embodiment of the compound of general formula I,  $R^3$ ,  $R^5$ , and  $R^6$  represent hydrogen; and  $R^4$  represents halogen, such as bromine.

15 In a further embodiment of the compound of general formula I,  $R^3$ ,  $R^5$ , and  $R^6$  represent hydrogen; and  $R^4$  represents phenyl substituted with trifluoromethyl, nitro or  $-CO-NHR^a$ ; wherein  $R^a$  is phenyl. In a further embodiment, the compound of general formula I is

*N*-4-Nitrophenyl-*N'*-[4-bromo-2-(1-*H*-tetrazol-5-yl)phenyl] urea;

20 *N*-3,5-Di(trifluoromethyl)phenyl-*N'*-[4-bromo-2-(1-*H*-tetrazol-5-yl)phenyl] urea;

*N*-3-Trifluoromethylphenyl-*N'*-[4-(3-nitrophenyl)-2-(1-*H*-tetrazol-5-yl)phenyl] urea;

*N*-3-Trifluoromethylphenyl-*N'*-[4-(4-anilincarbonylphenyl)-2-(1-*H*-tetrazol-5-yl)phenyl] urea;

*N*-3-Trifluoromethylphenyl-*N'*-[4-(4-trifluoromethylphenyl)-2-(1-*H*-tetrazol-5-yl)phenyl]

25 urea;

or a pharmaceutically acceptable salt thereof.

In a still further embodiment, the disease, disorder or condition that is responsive to inhibition of angiogenesis is selected from the group consisting of cancer, prostate cancer, lung cancer, breast cancer, bladder cancer, renal cancer, colon  
 30 cancer, gastric cancer, pancreatic cancer, ovarian cancer, melanoma, hepatoma, sarcoma, lymphoma, exudative macular degeneration, age-related macular degeneration, retinopathy, proliferative diabetic retinopathy, ischemic retinopathy, retinopathy of prematurity, neovascular glaucoma, corneal neovascularization, rheumatoid arthritis, and psoriasis.

35 In a special embodiment of the invention, the compound is

*N*-4-Nitrophenyl-*N'*-[4-bromo-2-(1-*H*-tetrazol-5-yl)phenyl] urea;  
*N*-3,5-Di(trifluoromethyl)phenyl-*N'*-[4-bromo-2-(1-*H*-tetrazol-5-yl)phenyl] urea;  
*N*-3-Trifluoromethylphenyl-*N'*-[4-(3-nitrophenyl)-2-(1-*H*-tetrazol-5-yl)phenyl] urea;  
*N*-3-Trifluoromethylphenyl-*N'*-[4-(4-anilinocarbonylphenyl)-2-(1-*H*-tetrazol-5-yl)phenyl]  
5 urea;  
*N*-3-Trifluoromethylphenyl-*N'*-[4-(4-trifluoromethylphenyl)-2-(1-*H*-tetrazol-5-yl)phenyl]  
urea;  
or a pharmaceutically acceptable salt thereof, and  
the treatment is an anti-metastatic treatment.

10

#### Methods of Preparation

The compounds for use according to the invention may be prepared by conventional methods for chemical synthesis, e.g. those described in WO 98/47879 or WO 00/24707.

15

#### Pharmaceutically Acceptable Salts

The active compound for use according to the invention may be provided in any form suitable for the intended administration. Suitable forms include pharmaceutically (i.e. physiologically) acceptable salts, and pre- or prodrug forms of the chemical  
20 compound of the invention.

Examples of pharmaceutically acceptable addition salts include, without limitation, the non-toxic inorganic and organic acid addition salts such as the hydrochloride, the hydrobromide, the nitrate, the perchlorate, the phosphate, the sulphate, the formate, the acetate, the aconate, the ascorbate, the  
25 benzenesulphonate, the benzoate, the cinnamate, the citrate, the embonate, the enantate, the fumarate, the glutamate, the glycolate, the lactate, the maleate, the malonate, the mandelate, the methanesulphonate, the naphthalene-2-sulphonate derived, the phthalate, the salicylate, the sorbate, the stearate, the succinate, the tartrate, the toluene-*p*-sulphonate, and the like. Such salts may be formed by  
30 procedures well known and described in the art.

Metal salts of a chemical compound of the invention include alkali metal salts, such as the sodium salt of a chemical compound of the invention containing a carboxy group.

#### **35 Pharmaceutical Compositions**

While the active compound for use in therapy according to the invention may be administered in the form of the raw chemical compound, it is preferred to introduce the active ingredient, optionally in the form of a physiologically acceptable salt, in a pharmaceutical composition together with one or more adjuvants, excipients, carriers,  
40 buffers, diluents, and/or other customary pharmaceutical auxiliaries.

In a preferred embodiment, the invention provides pharmaceutical compositions comprising the chemical compound for use according to the invention, or a pharmaceutically acceptable salt or derivative thereof, together with one or more pharmaceutically acceptable carriers therefor, and, optionally, other therapeutic and/or prophylactic ingredients, known and used in the art. The carrier(s) must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not harmful to the recipient thereof.

The pharmaceutical composition of the invention may be administered by any convenient route which suits the desired therapy. Preferred routes of administration include oral administration, in particular in tablet, in capsule, in dragé, in powder, or in liquid form, and parenteral administration, in particular cutaneous, subcutaneous, intramuscular, or intravenous injection. The pharmaceutical composition may be prepared by the skilled person using standard and conventional techniques appropriate to the desired formulation. When desired, compositions adapted to give sustained release of the active ingredient may be employed.

Further details on techniques for formulation and administration may be found in the latest edition of Remington's Pharmaceutical Sciences (Maack Publishing Co., Easton, PA).

The actual dosage depends on the nature and severity of the disease being treated, and is within the discretion of the physician, and may be varied by titration of the dosage to the particular circumstances of this invention to produce the desired therapeutic effect. However, it is presently contemplated that pharmaceutical compositions containing from about 0.1 to about 1000 mg of active ingredient per individual dose, preferably from about 1 to about 100 mg, are suitable for therapeutic treatments.

The active ingredient may be administered in one or several doses per day. Preferred ranges are from 10-200 mg/day p.o. administered in one or two doses, such as from 25-50 mg p.o. twice a day.

### 30 **Methods of therapy**

The invention also provides a method for the treatment, prevention or alleviation of a disease or a disorder or a condition of a living animal body, including a human, which disease, disorder or condition is responsive to inhibition of angiogenesis, and which method comprises administering to such a living animal body, including a human, in need thereof an effective amount of a compound of general formula I as defined above.

The diseases, disorders or conditions that are responsive to inhibition of angiogenesis include but are not limited to

- diseases, disorders or conditions that involve the proliferation of tumor cells, such as cancer, prostate cancer, lung cancer, breast cancer, bladder cancer, renal cancer, colon cancer, gastric cancer, pancreatic cancer, ovarian cancer, melanoma, hepatoma, sarcoma and lymphoma;
- 5 • ophthalmic angiogenesis related diseases, disorders or conditions, such as exudative macular degeneration, age-related macular degeneration, retinopathy, proliferative diabetic retinopathy, ischemic retinopathy (e.g. retinal vein or artery occlusion), retinopathy of prematurity, neovascular glaucoma, and corneal neovascularization; and
- 10 • rheumatoid arthritis, and psoriasis.

In a special embodiment, the disease, disorder or condition to be treated is a preneoplastic disease state. In a further embodiment, the treatment is an anti-metastatic treatment. In a still further embodiment, the disease, disorder or condition to be prevented is metastatic cancer.

15

### **Combined therapy**

The pharmaceutical composition for use according to the invention may include or may be used or administered in combination with one or more additional drugs useful for the treatment, prevention or alleviation of a disease responsive to inhibition  
20 of angiogenesis, such as compounds useful for anti-metastatic treatment. Such additional drugs include cytotoxic compounds, antimitotic compounds, and antimetabolites.

Examples of cytotoxic compounds (including cytotoxic alkylating agents) include carmustine (BCNU), fotemustin, temozolomide (temodal), ifosfamide, and  
25 cyclofosfamide.

Examples of antimitotic compounds include paclitaxel (taxol) and docetaxel.

An example of antimetabolites includes methotrexat.

Furthermore, the pharmaceutical composition for use according to the invention may be used or administered in combination with other treatments or therapies.  
30 Examples of other treatments or therapies include radiotherapy and surgery.

### **Test methods**

The efficacy of use of the compound according to the invention may be evaluated by standard *in vitro* and *in vivo* studies as e.g. those described below.

35

### **In vitro methods**

#### Cell-specificity assay: Incorporation of [<sup>3</sup>H]thymidine

Confluent cultures of HUVEC, fibroblasts, Mel 57 and T47D cells were detached  
40 by trypsin/EDTA solution, and allowed to adhere and spread at an appropriate cell

density on gelatine-coated dishes in M199-HEPES medium or DMEM-HEPES medium both supplemented with 10% heat-inactivated new born calve serum (NBCS) and penicillin/streptomycin. After 18 h the HUVEC and fibroblasts were stimulated with 2.5 ng/ml FGF-2 in M199-HEPES, penicillin/streptomycin, 10% NBCS and 0.1% DMSO in 5 duplicate wells, with or without the test compounds. The tumor cells were cultured in DMEM-HEPES supplemented with 10% NBCS, penicillin/streptomycin and 0.1% DMSO in duplicate wells, with or without the test compounds. After an incubation period of 48 h, a tracer amount (0.5  $\mu$ Ci/well) of [ $^3$ H]thymidine was added and the cells were incubated for another 6 h period. Subsequently, the cells were washed with PBS, [3H]-labelled 10 DNA were fixed with methanol, and precipitated in 5% trichloroacetic-acid, and finally dissolved in 0.5 ml 0.3 M NaOH and counted in a liquid scintillation counter.

#### Cell morphology and proliferation assay

One week before the assay, a vial with HUVEC (passage 1) was thawed and 15 cultured (after a split ratio of 1:3) to confluence (passage 2). The confluent culture of HUVEC was detached by trypsin/EDTA solution, and allowed to adhere and spread at cell density of 10, 50 and 100% confluency on gelatine-coated dishes in M199-HEPES medium supplemented with 10% heat-inactivated NBCS, 10% human serum and penicillin/streptomycin. After 18 h the HUVEC were preincubated with the test 20 compounds for 4 hours. Then the HUVEC were washed and restimulated in the absence or presence of the test compounds and the reference compounds with 2.5 ng/ml FGF-2 in M199-HEPES, penicillin/streptomycin, 10% NBCS, 10% human serum and 0.1% DMSO in triplicate wells for 5 (10% confluency) or 3 days (50 and 100% confluency), with or without the test compounds. The cell number was determined by image analysis 25 (P. Koolwijk, 2001)

#### Observations, analyses and measurements

All outcome measures were measured in singular, i.e. one measurement per culture well. The proliferation of HUVEC, fibroblast, Mel 57, and T47D tumor cells was 30 expressed as mean  $\pm$  range [3H]-thymidine incorporation (dpm) of duplicate wells.

The percentage of inhibition of FGF-2-induced HUVEC and fibroblast proliferation by the compounds was calculated as follows:

$$35 \quad \% \text{ inhibition} = \left( 1 - \frac{((\text{dpm, HUVEC}_{\text{FGF-2}}) - (\text{dpm, HUVEC}_{\text{FGF-2} + \text{compound}}))}{((\text{dpm, HUVEC}_{\text{FGF-2}}) - (\text{dpm, HUVEC}_{\text{control}}))} \right) \times 100\%$$

40	HUVEC <sub>control</sub>	= non stimulated HUVEC
	HUVEC <sub>FGF-2</sub>	= FGF-2 stimulated HUVEC
	HUVEC <sub>FGF-2 + compound</sub>	= FGF-2 stimulated HUVEC + test compound



The percentage of inhibition of the Mel 57 and T47D tumor cell proliferation by the compounds will be calculated as follows:

$$5 \quad \% \text{ inhibition} = \left( 1 - \frac{((\text{dpm, tumor cell}) - (\text{dpm, tumor cell} + \text{compound}))}{(\text{dpm, tumor cell})} \right) \times 100\%$$

tumor cell = NBCS stimulated tumor cell

tumor cell + compound = NBCS stimulated tumor cell + test compound

10

### In vivo methods

#### Mouse anti-angiogenesis assay

15 NMRI female mice (SPF Born:NMRI) weighing 25-27 g were obtained from M&B, Ejby, Lille Skensved, Denmark. They were housed in a facility where light was controlled on a 12 hour light-dark cycle. The room temperature and relative humidity recorded by a thermo-hygrograph showed values between 20.5-24.1°C and 40-67 %, respectively. The animals were fed a pelleted rodent diet (Altromin 1324, Brogård, Denmark) *ad libitum* and had free access to tap water. All animals were observed daily for clinical signs.

20 Slow-release pellets containing 400 ng of human basic fibroblast growth factor (Innovative Research of America, Florida, USA) were circular and with a diameter of 1.5 mm. The angiogenic peptide was guaranteed by the supplier to be released over a period of 10 days.

25 The mice were anaesthetized using inhalation anaesthesia (halothane/N<sub>2</sub>O and oxygen). The skin of the back was shaved using an electric shaver and the skin was disinfected using 70% ethanol. A 5 mm incision was made transversely in close proximity to the shoulder blades and a 2 cm pocket reaching caudally to the pelvic region was created by blunt dissection, carefully separating the skin from the fasciae. A polyurethane sponge with the dimension of 8x5x3 mm containing a slow-release pellet  
30 of 400 ng bFGF was placed at the caudal end of the pocket and the incision was closed by a single or double invert suture using Perma-Hand Seide 4/0 (Johnson&Johnson, Brussels, Belgium). The animals were treated by an analgesic subcutaneous injection of carprofen 2 mg/kg.

35 The angiogenic response was quantitated as previously described (Lichtenberg *et al.*, 1997, 1999 & 2002). Briefly, twenty minutes before euthanasia 1 µCi of <sup>125</sup>I-labelled immunoglobulin (Amersham, UK) in 50 µl of 0.9% NaCl was injected intravenously into a tail vein. The animals were euthanised by O<sub>2</sub>/CO<sub>2</sub> asphyxiation and the skin overlying the sponge implant was removed. The sponge implant with the pellet was placed in a plastic vial containing 4% formalin and the <sup>125</sup>I-activity was measured in a γ-counter.  
40 Differences in angiogenic response measured as <sup>125</sup>I-activity in cpm were assessed by

Student's t-test, grouped data, with  $P < 0.05$  regarded as statistically significant. Data was expressed as means  $\pm$  SEM.

#### Mouse metastasis assay

5 Female C57BL/6 mice were supplied and delivered by Charles River UK Ltd. The animals were approximately 6 weeks of age at the start of the study. The body weights at the start of dosing were in the range 10-21 g. The mice were housed in solid-bottomed plastic cages, containing wood shavings, in groups of up to 10. During acclimatization, the rooms and cages were cleaned at regular intervals to maintain  
10 hygiene. The mice were fed an expanded rodent diet ad libitum and allowed free access to mains tap water. The holding rooms had a 12 h light-dark cycle, and were air-conditioned by a system designed to maintain air temperature within the range  $20 \pm 3^\circ\text{C}$  (McKay, 2002).

Data was expressed as means  $\pm$  SEM and analysed using appropriate statistical  
15 methods. Statistical significance was assumed when  $P < 0.05$ .

### BRIEF DESCRIPTION OF THE DRAWINGS

The present invention is further illustrated by reference to the accompanying  
20 drawing, in which:

Fig. 1 shows the effects of Compound a on HUVEC (O), fibroblast ( $\blacktriangle$ ) and tumor cell (Mel 57( $\bullet$ ) and T47D( $\nabla$ )) proliferation;

Fig. 2 shows the effects of Compound a on 10%, 50% and 100% confluent monolayers of HUVEC. Solid symbols and bars: continuous conditions; open symbols  
25 and bars: preincubated conditions.

### EXAMPLES

The invention is further illustrated with reference to the following examples, which are not  
30 intended to be in any way limiting to the scope of the invention as claimed. The examples describe test results for the compounds *N*-4-Nitrophenyl-*N*'-[4-bromo-2-(1-*H*-tetrazol-5-yl)phenyl] urea (compound a) and *N*-3,5-Di(trifluoromethyl)phenyl-*N*'-[4-bromo-2-(1-*H*-tetrazol-5-yl)phenyl] urea (compound b).

#### 35 Example 1

##### **Compound a tested in the *in vitro* cell specificity assay**

A difference in cell-specificity was observed of Compound a (see Fig. 1). The compound was a more potent inhibitor of the bFGF-induced HUVEC and fibroblast proliferation when compared to the NBSC-induced Mel 57 and T47D proliferation.

**Example 2****Compound a tested in the *in vitro* cell morphology and proliferation assay**

To investigate the mode of action of the compound on proliferating and non-proliferating HUVEC, experiments were performed on 10%, 50% and 100% confluent  
5 monolayers of HUVEC. The 10% and 50% confluent HUVEC monolayers represent the status of angiogenic endothelial cells able to proliferate and migrate when stimulated. The 100% HUVEC monolayers represent the quiescent character of the endothelial cells in the existing blood vessel. The 4-hour preincubation experiments were performed to be able to discriminate between general toxicity and the induction of apoptosis.

10 Compound a was not able to inhibit HUVEC proliferation significantly when added for the 4-hour preincubation period and then removed for the rest of the stimulation period (see Fig. 2). No signs of any cytotoxicity (cell death indicated by the observation of floating cells in the media) was observed during this period. In addition, there was no difference in cell death or any delay of cell growth by the preincubation period during the  
15 rest of the 3-day or 5-day incubation period.

However, when the incubation was performed in the continuous presence of the compound, there was a clear inhibitory effect on the proliferation of the cells at the 10% confluence HUVEC monolayers. This effect was observed at the two highest concentrations of the compound, but not at lower concentrations. The compound  
20 induced inhibition of HUVEC proliferation was also slightly observed at the 50% confluent monolayers (see Fig. 2). There was no effect of the compound on the 100% HUVEC monolayers.

This non-cytotoxicity of the compound on the 100% confluent monolayers was confirmed by the fact that there was no change of HUVEC morphology and the amount  
25 of floating dead cells in the culture medium observed during the culture periods with the compound.

**Example 3****Compounds a and b tested in the *in vivo* mouse anti-angiogenesis assay**

30 Three separate experiments were conducted: Two experiments were performed with Compound a and one experiment was performed with Compound b. In the first two experiments with Compound a, 3 groups each comprising 5 animals was treated orally with Compound a at the dose levels of 0 (saline), 5 and 10 mg/kg/day (experiment 1) and with 20 and 40 mg/kg/day (experiment 2). In the third experiment, 3 groups each  
35 comprising 6 animals was treated orally with the vehicle (saline) and 80 mg/kg/day of Compound a. In all experiments the animals were treated from Days 3-9 and killed at day 10.

Compound a elicited a dose-response relationship at all doses. At 10 and 40 mg/kg/day a significant inhibition of the neovascularisation of 37% and 48% was obtained (see Table 1). A dose of 5 mg/kg/day appeared to be the No Effect Level (NOEL).

Compound b at a dose of 80 mg/kg/day inhibited the angiogenesis response of approximately 60% compared to vehicle treated animals. The selected dose levels were well tolerated by the mice; no signs of toxicity or changes of the body weight gain were observed (data not shown).

**Table 1**

The effect of Compounds a and b on the neovascularisation in mice (expressed as percent inhibition)

Dose mg/kg→ Treatment ↓	5	10	20	40	80
Compound a	15%	37%*	28%	48%*	
Compound b					61%*

\*  $P < 0.05$ , compared to vehicle (t-test)

#### Example 4

##### Compounds a and b tested in the *in vivo* mouse metastasis assay

Two separate studies were conducted: One was performed with Compound a (experiment 1) and one with Compound b (experiment 2). In each study there were 4 treatment groups. The treatment groups were as follows:

Group	Treatment	Dose
1	Untreated Control	-
2	Vehicle Control	20 ml/kg p.o.
3	Compounds a or b	60 mg/kg p.o.
4	Compounds a or b	80 mg/kg p.o.

Treatments for groups 2-4 were administered orally, by gavage. The dose volume used was 20 ml/kg for groups 2 and 4 and 15 ml/kg for group 3.

C57BL/6 mice were injected intravenously, via a tail vein, with 0.1 ml of a suspension of B16/F10 melanoma cells (approximately  $3 \times 10^5$  cells/mouse) on day 0. With the exception of the untreated group, animals were dosed orally, by gavage, or intravenously, according to their allocated treatment group once daily, from day -2 to day 10 (13 administrations). The animals were killed on day 14 (14 days after injection of the tumour cells). The lungs from each animal were removed and weighed prior to fixing in Bouin's solution. After fixation, the number of colonies on the surface of each set of lungs was counted by eye and these data were used for statistical analysis.

Oral administration of Compound a, at doses of 60 and 80 mg/kg, resulted in a significant reduction of 17% and 21%, respectively in the number of melanoma lung colonies when compared to vehicle treated mice (see Table 2).

- 5 Oral administration of Compound b, at similar doses (60 and 80 mg/kg), produced a significant reduction of 36% and 44%, respectively in the number of melanoma lung colonies compared to vehicle treated mice (see Table 3).

**Table 2**

- 10 The effect of Compound a on the development of B16 melanoma lung colonies in C57BL/6 mice

Group	Treatment	Colony Count	% Reduction
1	Untreated Control	-	-
2	Vehicle Control (20 ml/kg)	78.29 ± 3.56	-
3	Compound a (60 mg/kg)	64.68 ± 2.65*	17.38
4	Compound a (80 mg/kg)	61.85 ± 2.89*	21.00

\*  $P < 0.01$ , compared to vehicle (Kruskal-Wallis and Dunnett's test)

15 **Table 3**

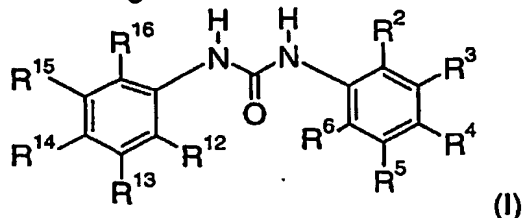
The effect of Compound b on the development of B16 melanoma lung colonies in C57BL/6 mice

Group	Treatment	Colony Count	% Reduction
1	Untreated Control	-	-
2	Vehicle Control (20 ml/kg)	65.40 ± 7.90	-
3	Compound b (60 mg/kg)	41.55 ± 9.55*	36.45
4	Compound b (80 mg/kg)	36.65 ± 5.82*	43.97

\*  $P < 0.01$ , compared to vehicle (Kruskal-Wallis and Dunnett's test)

**CLAIMS:**

1. The use of a compound of general formula I

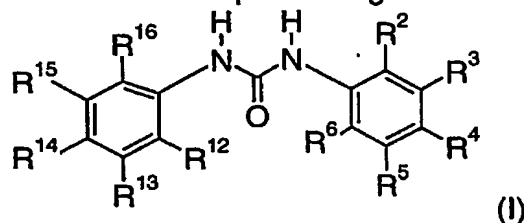


- 5 or a pharmaceutically acceptable salt thereof  
 wherein  $R^2$  represents tetrazolyl;  
 $R^3$ ,  $R^4$ ,  $R^5$ ,  $R^6$ ,  $R^{12}$ ,  $R^{13}$ ,  $R^{14}$ ,  $R^{15}$ , and  $R^{16}$  independently of each other represent  
 hydrogen, halogen, trifluoromethyl, nitro, or phenyl;  
 which phenyl is optionally substituted with halogen, trifluoromethyl, nitro, or  $-CO-NHR^a$ ;  
 10 wherein  $R^a$  is hydrogen, alkyl, or phenyl;  
 for the manufacture of a pharmaceutical composition for the treatment, prevention or  
 alleviation of a disease or a disorder or a condition of a mammal, including a human,  
 which disease, disorder or condition is responsive to inhibition of angiogenesis.
- 15 2. The use according to claim 1, wherein  
 $R^3$ ,  $R^5$ , and  $R^6$  represent hydrogen; and  
 $R^4$  represents halogen.
3. The use according to claim 1, wherein  
 20  $R^3$ ,  $R^5$ , and  $R^6$  represent hydrogen; and  
 $R^4$  represents phenyl substituted with trifluoromethyl, nitro or  $-CO-NHR^a$ ;  
 wherein  $R^a$  is phenyl.
4. The use according to claim 1, wherein the compound is  
 25 *N*-4-Nitrophenyl-*N'*-[4-bromo-2-(1-*H*-tetrazol-5-yl)phenyl] urea;  
*N*-3,5-Di(trifluoromethyl)phenyl-*N'*-[4-bromo-2-(1-*H*-tetrazol-5-yl)phenyl] urea;  
*N*-3-Trifluoromethylphenyl-*N'*-[4-(3-nitrophenyl)-2-(1-*H*-tetrazol-5-yl)phenyl] urea;  
*N*-3-Trifluoromethylphenyl-*N'*-[4-(4-anilinocarbonylphenyl)-2-(1-*H*-tetrazol-5-yl)phenyl]  
 urea;  
 30 *N*-3-Trifluoromethylphenyl-*N'*-[4-(4-trifluoromethylphenyl)-2-(1-*H*-tetrazol-5-yl)phenyl]  
 urea;  
 or a pharmaceutically acceptable salt thereof
5. The use according to claim 1, wherein the disease, disorder or condition that is  
 35 responsive to inhibition of angiogenesis is selected from the group consisting of cancer,

prostate cancer, lung cancer, breast cancer, bladder cancer, renal cancer, colon cancer, gastric cancer, pancreatic cancer, ovarian cancer, melanoma, hepatoma, sarcoma, lymphoma, exudative macular degeneration, age-related macular degeneration, retinopathy, proliferative diabetic retinopathy, ischemic retinopathy, 5 retinopathy of prematurity, neovascular glaucoma, corneal neovascularization, rheumatoid arthritis, and psoriasis.

6. The use according to claim 1, wherein the compound is  
*N*-4-Nitrophenyl-*N'*-[4-bromo-2-(1-*H*-tetrazol-5-yl)phenyl] urea;  
 10 *N*-3,5-Di(trifluoromethyl)phenyl-*N'*-[4-bromo-2-(1-*H*-tetrazol-5-yl)phenyl] urea;  
*N*-3-Trifluoromethylphenyl-*N'*-[4-(3-nitrophenyl)-2-(1-*H*-tetrazol-5-yl)phenyl] urea;  
*N*-3-Trifluoromethylphenyl-*N'*-[4-(4-anilincarbonylphenyl)-2-(1-*H*-tetrazol-5-yl)phenyl]  
 urea;  
*N*-3-Trifluoromethylphenyl-*N'*-[4-(4-trifluoromethylphenyl)-2-(1-*H*-tetrazol-5-yl)phenyl]  
 15 urea;  
 or a pharmaceutically acceptable salt thereof, and  
 the treatment is an anti-metastatic treatment.

7. A method of treatment, prevention or alleviation of a disease or a disorder or a  
 20 condition of a living animal body, including a human, which disorder, disease or  
 condition is responsive to inhibition of angiogenesis, comprising the step of  
 administering to such a living animal body, including a human, in need thereof a  
 therapeutically effective amount of a compound of general formula I



- 25 or a pharmaceutically acceptable salt thereof  
 wherein  $R^2$  represents tetrazolyl;  
 $R^3$ ,  $R^4$ ,  $R^5$ ,  $R^6$ ,  $R^{12}$ ,  $R^{13}$ ,  $R^{14}$ ,  $R^{15}$ , and  $R^{16}$  independently of each other represent  
 hydrogen, halogen, trifluoromethyl, nitro or phenyl  
 which phenyl is optionally substituted with halogen, trifluoromethyl, nitro, or  $-CO-NHR^a$ ;  
 30 wherein  $R^a$  is hydrogen, alkyl, or phenyl.

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<sup>3</sup>H-thymidine incorporation (% of 0  $\mu$ M)

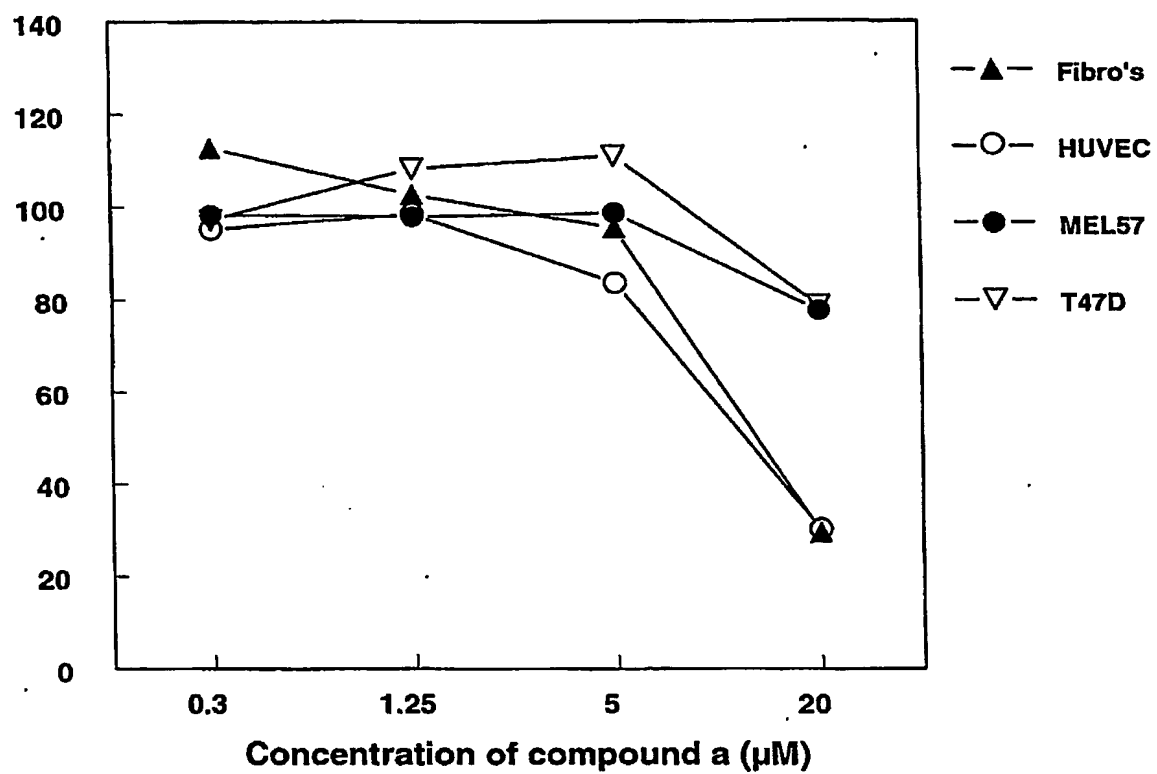


Fig. 1



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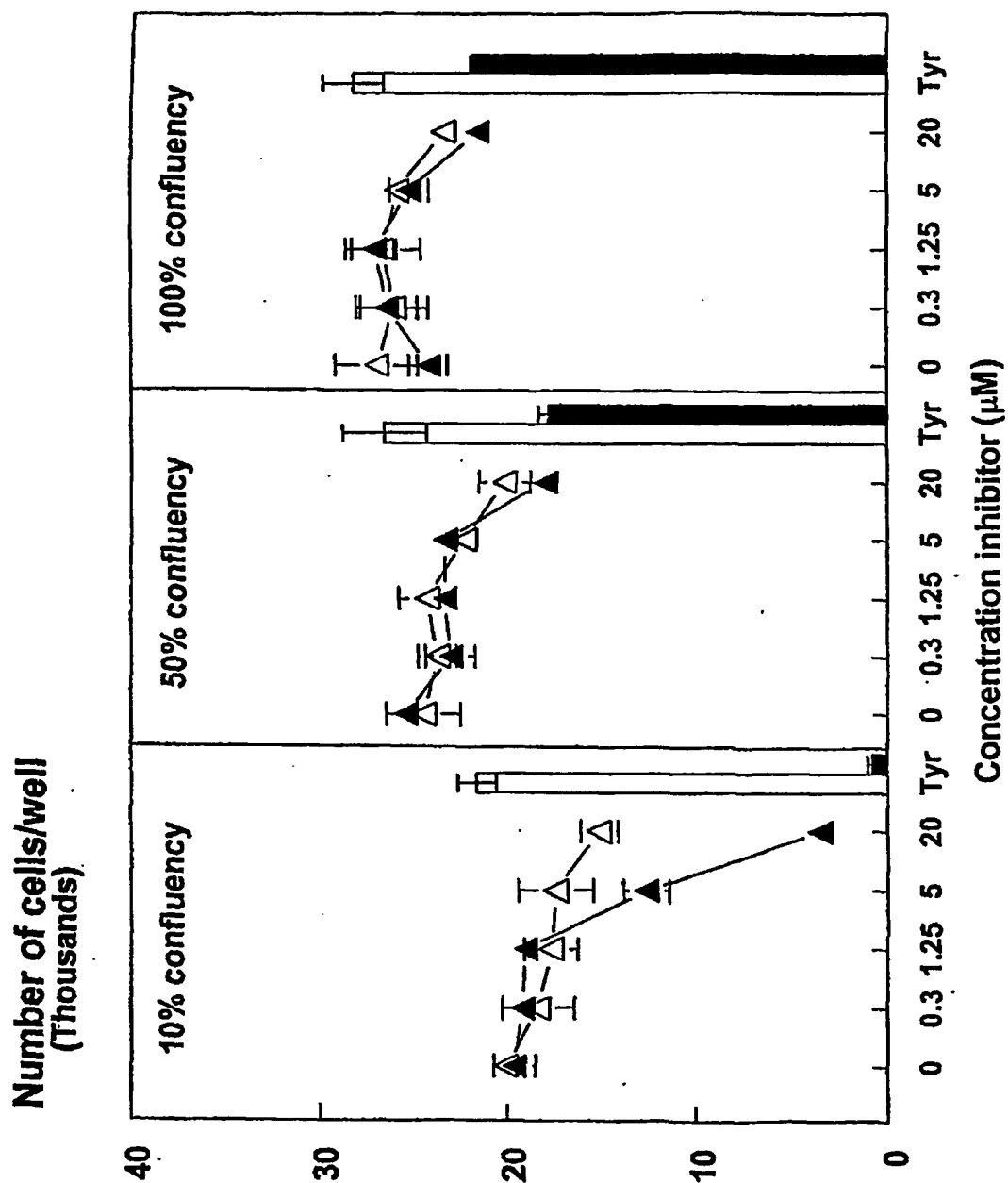


Fig. 2